2004

Isolation of Aflatoxin-Free Lutein from Aflatoxin-Contaminated Corn

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ISOLATION OF AFLATOXIN-FREE LUTEIN FROM AFLATOXIN-CONTAMINATED CORN

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Evdokia Menelaou
B.S., The University of Louisiana at Monroe, 2002
August 2004

DEDICATION

To my parents Marios and Charoulla Menelaou and my brother Melios Menelaou for their love, remarkable support, and encouragement in fulfillment of this study.

ACKNOWLEDGMENTS

I am profoundly thankful to Dr. Jack N. Losso, my major professor and mentor, who guided me and supported me to the full extent throughout this study. I would like to express my gratitude for his guidance, patience, understanding, and his constructively critical eye. His knowledge has been extremely helpful for the completion of this study. He was a great professor and friend and he continuously encouraged me for the better. I would also like to thank the other members of my committee, Dr. Joan M. King and Dr. Manjit S. Kang, for the assistance they provided for this project and Dr. Janet G. Simonson for her help in my laboratory work.

I would also like to give special thanks to Dr. Masahiro Ogawa for his assistance when I first started on this project and his great mentor abilities. He was always willing to provide his technical knowledge and ability in troubleshooting and he has been a great friend above all.

Most importantly, I would like to thank my friends and my family for their great support and encouragement throughout this study. My family has been consistently supportive and understanding of my effort and my friends have always been by my side in all the difficult moments.

Very special thanks go out to my friends Rishipal R. Bansode and Armen Khachatryan for their support, inspiration, and input in completing this work. Their knowledge has been of great help at all times. Their advice and encouragement have been extremely helpful.

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ABSTRACT

Lutein (3,3'-dihydroxy-alpha-carotene) has been identified by the Age-Related Eye Disease Study (AREDS) of the National Institute of Health (NIH) as a dietary compound with the ability to delay the onset and/or progression of age-related and/or diabetes-related vision loss. Lutein can also be useful in the prevention of other angiogenic diseases such as breast and colon cancer.

Although marigold (*Tagetes erecta*) flowers are an excellent source of lutein, corn (*Zea mays*) has been identified as a very economical source of lutein because more value-added products, such as lutein, oil, and zein (known for its anti-microbial and anti-hypertensive activities) can be isolated from corn than marigold flowers. However, aflatoxin-contaminated corn has very low economic value to farmers and is banned for use in human food chain. The objective of this research was to isolate aflatoxin-free lutein from aflatoxin-contaminated corn.

Aflatoxin-contaminated corn was fractionated for lutein using commercially available solvents. Aflatoxins in the aflatoxin-lutein mixture were converted into a water-soluble molecule and were displaced from the lipid environment. Extraction and quantification of aflatoxin in the aqueous and lutein-containing lipid phase were carried out using the AOAC multifunctional column method involving solid phase extraction and HPLC, respectively. Aflatoxins B_1 and B_2 were identified at 4888 and 368 ppb, respectively in the untreated aflatoxins-lutein extract. However, no peaks associated with either aflatoxin B_1 or B_2 were detected in the lipid phase containing lutein following aflatoxins displacement. Lutein concentration and stability following aflatoxins displacement was determined by HPLC. The HPLC results indicated the presence of one

peak eluted at 21.0 minutes. Spiking with standard lutein confirmed the identity and stability of lutein isolated from aflatoxins-contaminated corn.

This study has shown that corn growers and processors may generate additional income from aflatoxin-contaminated corn. The overall significance of this research is that, if approved by FDA, corn growers can still sell aflatoxins-contaminated corn at a competitive price since almost all the value-added products from corn can be recovered aflatoxins-free and more lutein will be available for disease prevention.

CHAPTER 1
INTRODUCTION

Corn (*Zea mays*) is a popular and widely consumed food and feed commodity in many communities throughout the world. Corn susceptibility to aflatoxin contamination, however, provides a potential health hazard to both human consumers and animals (Piedade et al., 2002). The fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, that produce aflatoxins before and during harvesting, processing, and storage (Scudamore, 1998), can infect important food and feed crops under favorable conditions of temperature (>90 ^{o}F) and humidity (>80%). *Aspergillus flavus* and *A. parasiticus* are soil-borne fungi and grow on both living and decaying plant tissues.

Mycotoxins are secondary metabolites produced by fungi in foods and feeds, which, on ingestion, can result in illness or death of animals, including humans (McLean and Dutton, 1995). The degree of toxicity may vary depending upon the amount of contamination and the presence of different metabolites of aflatoxins (Uraguchi and Yamazaki, 1978). There are four naturally occurring aflatoxin metabolites produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. These are named aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2. *Aspergillus flavus* produces only AFB_1 and AFB_2, and *Aspergillus parasiticus* produces AFB_1, AFB_2, AFG_1, and AFG_2. Aflatoxin B_1, the most potent of mycotoxins, is usually found in the highest concentrations and causes primary liver cancer (PLC) (McLean and Dutton, 1995).

Corn is of great importance because of its oil, starch, and protein content. Lutein and zeaxanthin are plant pigments that belong to the group of carotenoids. The significance of human consumption of foods rich in lutein is that because humans are not

capable of synthesizing carotenoids *in vivo*; the presence of lutein in human tissue is solely dependent upon dietary origin. Lutein is found in green leafy vegetables (e.g., spinach, broccoli, green beans) and fruits. In corn, lutein is mostly found in the horny endosperm and the total xanthophyll content in whole corn is 11-39 mg/kg and the lutein content alone is 7-30 mg/kg (Johnson, 2002). Zeaxanthin is a structural isomer of lutein and is similar to lutein relative to food sources, human metabolism, and tissue storage (Johnson, 2002). Both lutein and zeaxanthin are also called xanthophylls or macula pigments. They are the major xanthophylls found in the macula of the inner retina of the human eye (Berstein, 2002). They act as antioxidants and blue-light filters because of their high absorptivity and yellow color. There is epidemiological evidence that the amount of macular pigment (lutein and zeaxanthin) in the eye is in inverse relation to the incidence of age-related macular degeneration (AMD), a degenerative process that is one of the major causes of blindness in the elderly and diabetic patients (Krinsky et al., 2003). Lutein by its protective mechanism can reduce the stress in the retina, thus reducing the risk of AMD.

Corn is a basic ingredient in human food and animal feed. Corn for grain production in 2003 was 257 billion Kg or 10.1 billion bushels (USDA, 2003). About 57% of corn crop produced in the USA was used for feed, which is about 145 billion Kg. An estimated 45.8 billion Kg was used for exports, which is about 20% of total corn crop production for 2003 (USDA, 2003). About 24% of corn crop was used for seed, food, and industrial purposes in 2003 (Troyer and Good, 2005). Due to its nutritional composition, corn is a good substrate for fungi development that may cause production of toxic substances, thus, limiting corn marketability and causing economic losses (Piedade et al.,

2002, Betran et al., 2002). The FDA requires that products contaminated with aflatoxins levels higher than 20 ppb not be used for human consumption (FDA, 2000).

The objective of the present project was to extract aflatoxin-free lutein from aflatoxin-contaminated corn. Previous research has been conducted to either prevent the level of aflatoxin contamination before harvest or detoxify aflatoxin-contaminated corn after harvesting (Zuber et al., 1987). No previous research was performed in achieving the isolation of lutein, which has a great health benefit, from aflatoxin-contaminated corn after harvesting. The impact of aflatoxin contamination is pronounced on the agricultural economy in drought-stricken years, with estimated losses ranging in the hundreds of millions dollars (Kang and Moreno, 2002). Losses for farmers can be in the form of yield, nonmarketable grain, restricted markets, increased transportation costs, discounts, increased costs of drying and selling, and inability to obtain loans on stored grain (Nichols, 1983). The present study was initiated to make use of aflatoxin-contaminated corn after harvesting rather than discarding this traditional agricultural commodity. The production rates of corn suggest that any solution found to make use of aflatoxin-contaminated corn may have a wide range of economic, health, and social implications in the USA and around the world.

CHAPTER 2
LITERATURE REVIEW

I. Corn as a Source of Lutein

1. Introduction

People's interest in long-term health maintenance has been increasing very rapidly and this has led to the development of new food products with nutritional benefits. Carotenoids are epidiomiologically linked with the prevention of several chronic and degenerative diseases in humans, thus leading to a significant demand for their isolation from natural sources for health-enhancing benefits (Johnson, 2002).

Xanthophylls are pigments of the carotenoid family, which are one of the most abundant phytochemical groups naturally found in plants (Moros et al., 2002). Xanthophylls include astaxanthin, lutein, and zeaxanthin, of which the latter two are the major carotenoids found in fruits, vegetables, and the human retina of the eye (Dagnelie et al., 2000). These xanthophylls have a major protective role in the human retina because of their photoreception and very high absorptivity properties, thus leading to blue-light filtration before reaching the back of the eye (Mares-Perlman et al., 2001). Another important property of carotenoids is their high oxidizing potential that protects plants and the retina of the eye from scavenging peroxyl radicals and quenching reactive oxygen species (Rapp et al., 2000). The human body is not capable of synthesizing carotenoids *in vivo*, thus, their presence in human tissues is entirely of dietary origin, although man is capable of modifying some of them to some extent (Granado et al., 2003).

2. Sources of Lutein

Because humans do not synthesize lutein *in vivo*, it should be obtained from outside sources. The major source of lutein intake is diet. Spinach and kale are the most

lutein-abundant foods (Table 1) (Johnson, 2002). However, other major dietary sources include egg yolk, broccoli, brussels sprouts, green peas, corn, and generally dark leafy vegetables, such as sweet potato leaves (Krinsky et al., 2003).

Table 1. Lutein/Zeaxanthin Content of Foods

Food (cooked)	Content (mg/100g wet wt)*
Broccoli	2.2
Brussels sprouts	1.3
Corn	1.8
Green peas	1.4
Kale	15.8
Spinach	7.1
Spinach (raw)	11.9

**Edible portion.*
Adapted from Johnson, 2002.

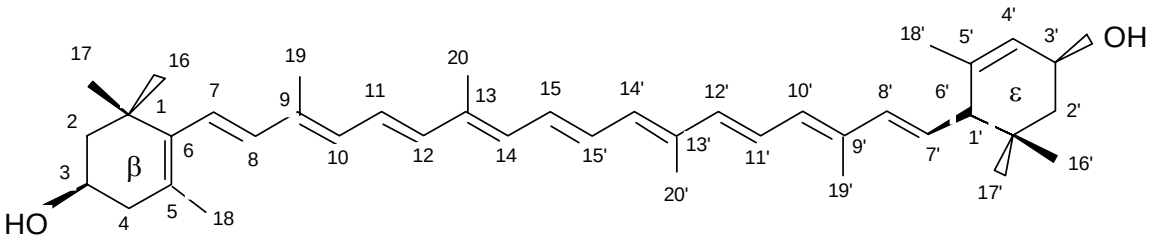
According to Krinsky et al. (2003), egg yolks have relatively low values of lutein content, but lutein and zeaxanthin obtained from this food are highly bioavailable. In relation to food sources, human metabolism, and tissue storage, lutein and zeaxanthin are similar. Alternative sources of lutein include supplement products that contain lutein, or lutein diester in amounts of 3-20 mg/capsule (Johnson, 2002).

3. Physical and Chemical Properties of Lutein

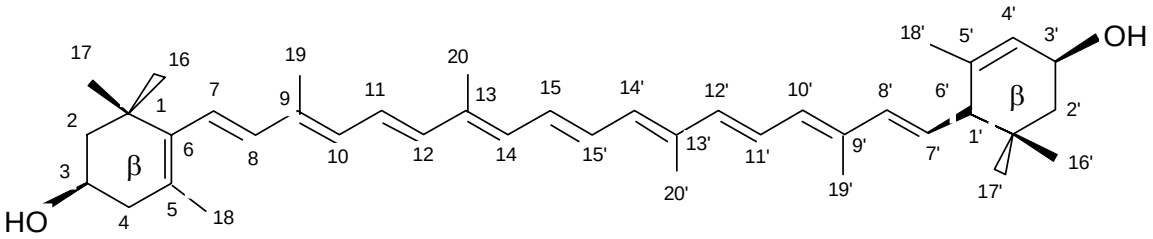
Lutein and zeaxanthin have distinguishing characteristics that account for their biological role in light energy collection and photoprotection (Beaty et al., 1999). From a simplistic view, chemical function is dependent upon structure. The chemical structure embodies the physical shape, charge distribution, and energy levels of a molecule. The characteristic feature of the xanthophyll structure is the alternating double and single bonds that form the central part of the molecule (Figure 1). It is this feature that gives xanthophylls their shape, chemical reactivity, and light-absorbing properties that result in various shades of red, yellow, and orange colors (Moros et al., 2002).

Lutein and zeaxanthin are both isomeric dihydroxy-carotenoids with the ionone ring systems being substituted at both the 3 and 3' carbon. In zeaxanthin, the ionone rings are β types and the β-ionone ring double bond is located between the C_5 and C_6 carbons (Goodwin, 1980). The carbons sharing the two hydroxyl groups share an identical R stereochemical configuration in the most common form of zeaxanthin (3R, 3R'-zeaxanthin), most commonly found in higher plants (Krinsky et al., 2003). Lutein has both β-ionone and ε-ionone rings and the presence of hydroxyl groups at both 3 and 3' carbons implies that a similarity exists in physical properties between lutein and zeaxanthin. The ε-ionone ring has $C_4 - C_5$ double bond and an allylic 3'-hydroxyl group (Andrewes et al., 1974)

The most predominant natural stereoisomer form of lutein is (3R, 6'R, 3'R)-β,ε-carotene-3, 3-diol (Landrum and Bone, 2001). The ε-ring hydroxyl group is oppositely directed in respect to the hydroxyl group in the β-ionone ring. The 3'-hydroxyl of the zeaxanthin ring projects forward from the planar surface of the page whereas the 3'-hydroxyl of lutein is extended back away from the plane of the page. Another significant characteristic resulting from the presence of the ε-ionone ring in lutein is that C_6 carbon, which is attached to the polyene chain, is a stereocenter. The consequence of a tetrahedral carbon at C_6 in the ε-ionone ring is that a slight rotation about the C_6-C_5 bond can relieve strain caused by the C_{18} methyl group. This is not possible for the β-ionone ring where the double bond constrains the geometry between C_6- C_5. (Figure 1). The result of this difference between ring substitutions is that the β-hydroxyl groups in zeaxanthin and lutein are directed in an axial direction whereas that of lutein ε group is directed equatorial with respect to the ring plane (Krinsky et al., 2003).



Lutein, (3R, 3’R, 6’R)-β,ε-caroten-3,3’-diol



Zeaxanthin, (3R, 3’R)-β,β-caroten-3,3’-diol

Figure 1. Structures of Lutein and Zeaxanthin.

The most predominant characteristic of these carotenoids is the presence of nine or more conjugated carbon-carbon double bonds within the structure, which allows susceptibility to light, oxygen, heat, and acid degradations (Updike and Schwartz, 2003). These conjugated double bonds have the ability to quench singlet oxygen with increasing activity depending on the number of conjugated double bonds (Bohm et al., 2002). This unique structure of lutein and zeaxanthin allows them to function as primary antioxidants in biological systems by scavenging peroxyl radicals. Oxidation of alcohols readily occurs *in vivo* and *in vitro* to produce carbonyl functional groups. The alcohols found in lutein and zeaxanthin produce ketones upon oxidation. In lutein, the alcohol of the ε-ionone ring is allylic to the double bond of the ring, thus is more readily oxidized than the β-ring hydroxyl groups of both lutein and zeaxanthin (Landrum and Bone, 2001). The polyene chain of carotenoids can also be oxidized by reaction with a peroxyl radical.

Loss of a single electron from the conjugated chain will result in the formation of a cation radical. A double-bond isomerization may occur, converting lutein directly to *meso*-zeaxanthin. Generally, carotenoids are believed to function as antioxidants because of their ability to form resonance stabilized radical cations or radical adducts, which are not capable of participating in autoxidation reactions (Mortensen and Skibsted, 2000).

The presence of hydroxyl groups makes lutein and zeaxanthin noticeably more polar than their respective analogs of α- and β- carotene. This is distinctively demonstrated by their relative retention times on both normal and reverse-phase chromatographic columns where the difference in retention times is due primarily to polarity. Lutein is soluble in nonpolar or dipolar solvents given in Table 2.

Table 2. Some Physical Properties of Lutein and Solubility Data in Organic Solvents.

Physical Properties	
Molecular formula	$C_{40}H_{56}O_2$
Molecular weight	568.85
Melting point	183-190 ^{o}C
Appearance	Yellow prisms with metallic luster
Stability	Unstable to light and oxygen
	Stable at -20 ^{o}C and under nitrogen atmosphere
Solubility in water	Insoluble

Solubility in Organic Solvents			
Solvent	Solubility (mg/L)	Solvent	Solubility (mg/L)
Acetone	800	Ethyl acetate	800
Acetonitrile	100	Ethyl ether	2000
Benzene	600	Hexane	20
Chloroform	6000	2-Propanol	400
Cyclohexane	50	Methyl alcohol	200
Cyclohexanone	4000	Methyl tert butyl ether	2000
Dichloro methane	800	Tetrahydrofuran	8000
Diemthyl formamide	1000	Toluene	500
Ethyl alcohol	300		

Adapted from Antony and Shankaranarayana, 2001.

In natural systems, lutein and zeaxanthin are found in many different chemical environments. In foods, lutein can be found either in its free form, bound to proteins, or esterified as a monoester or di-ester (Granado et al., 2003). Most of the lutein and zeaxanthin found in plant leaves is bound on proteins. In humans and higher animals, lutein and zeaxanthin are found in lipophilic tissues where they are transported by the lipoproteins, which is similar to cholesterol transport (Krinsky et al., 2003).

The presence of the conjugated polyene chain gives lutein and zeaxanthin the ability to absorb light. The extent of conjugation of the polyene chain is broadly related to the wavelength absorption band. Lutein has an absorption maximum of 445 nm in ethanol whereas that of zeaxanthin is 451nm.

4. Lutein in Health and Disease

In recent years, several epidemiological studies have supported an inverse relationship between lutein and the risk of certain eye disorders and other degenerative diseases. In 1945, George Wald was the first to demonstrate that macular pigment exhibited a distinct carotenoid spectrum and concluded that xanthophyll families found in green leafy vegetables were the origin of this pigment (Beaty et al., 1999).

Age-related macular degeneration (AMD) is a degenerative disease in the macula of the eye and is the leading cause of blindness in individuals aged 55 years or older in the western world. AMD affects the *macula lutea*, also known as fovea of the human eye, the central yellow spot of the retina in humans, which is responsible for sight, and sharp and detailed vision (Dachtler et al., 2001). The *macula lutea*, is composed of the hydroxyl-carotenoids lutein and zeaxanthin, which are responsible for the yellow color of the macula (Hammond et al., 2001). Bone et al. (1988) were the first to demonstrate by

high performance liquid chromatography (HPLC) that there were two xanthophylls, lutein and zeaxanthin, actually present in the macula.

Macular pigment acts as an effective filter against damaging blue light, which reduces chromatic aberration in the eye (Beaty et al., 2001). Filtration of short wavelength light by macular pigment may also prevent photochemical damage to cones and retinal pigment epithelium (RPE) in the fovea (Rapp et al., 2000). Macular pigment other than an optical filter can also act as an antioxidant. In the retina, the generation of reactive oxygen species (ROS) can occur as byproducts of cellular metabolism or as the result of photochemical reactions. There is sufficient evidence to suggest that lutein and its structural isomer, zeaxanthin, could reduce the risk of AMD. The antioxidant properties of these retinal carotenoids, lutein and zeaxanthin, have been extensively investigated and they include the ability to quench the triplet state of photosensitizers and singlet oxygen reactivity with free radicals, and chain- breaking antioxidant properties to retard the peroxidation of membrane phospholipids (Beaty et al., 1999). Another mechanism by which lutein and/or zeaxanthin might protect against AMD is their ability to absorb light before it reaches the back of the retina (Mares-Perlman et al., 2001).

Epidemiological and observational studies have shown that concentration of macular carotenoids can be manipulated by dietary intake of lutein and zeaxanthin due to their significant protective role in lowering the risk of AMD (Humphries and Khachik, 2003). The Eye Disease Case-Control Study Group (1993) found that the upper quartile of the population in their study consumed greater than 5.6 mg of lutein and zeaxanthin per day and had a lowered risk for the occurrence of late-stage AMD. According to the study of Sommerburg et al. (1998), consumption of fruits and vegetables of various

colors would increase the intake of lutein and zeaxanthin. However, their study did not investigate the effectiveness of consumption of lutein and zeaxanthin in reducing the risk of AMD. Bone et al. (2001) determined the concentration of lutein and zeaxanthin in donor eyes with and without AMD. Their study reinforced earlier epidemiological studies that showed relevance between low levels of lutein and zeaxanthin in the diet or serum and increased risk of neovascular AMD.

Research is currently performed investigating the association of carotenoid intake and cancer. Epidemiological studies have shown that there is an inverse relation between the risk of cancer and the consumption of green and yellow vegetables and fruits, in which lutein is present (Nishino et al., 2000). The effect of lutein on lung carcinogenesis was investigated and it was shown that lutein has anti-tumor promoting ability. This animal cell study demonstrated that treatment with lutein has a tendency to decrease the formation of lung tumors. Slattery et al. (2000) showed that lutein was the only carotenoid that appeared to be inversely associated with colon cancer. The results suggest that the protective effect of lutein against colon cancer is the same in both men and women. Lutein intake showed an inverse association for all subjects, especially younger individuals diagnosed with cancer. Study by Nkondjock and Ghadirian (2004), showed that reduced risk of colon cancer was also associated with the consumption of fruits and particularly vegetables. It has been shown that increase in dietary intake of lutein and zeaxanthin by 10% is associated with a 2.4% increase in serum lutein concentration. The above studies reinforce the consumption of plant foods, especially vegetables, for cancer prevention.

Despite the increasing amount of literature dealing with different aspects in humans, there is still no definitively established, physiologically significant threshold for lutein in serum above which protection or prevention against chronic diseases is ensured or provided. The percentage of the population consuming lutein-containing supplements is rapidly increasing and a variety of lutein-fortified foods could become commercially available (Losso et al., 2004).

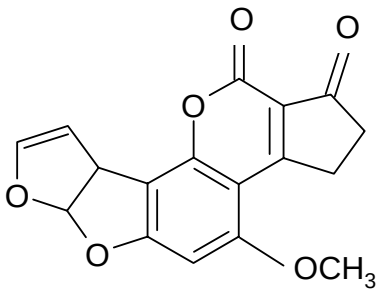
II. Aflatoxins

1. Introduction

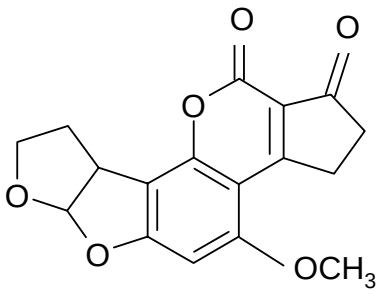
There are approximately 20 compounds designated as aflatoxins and the term usually refers to fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Scudamore, 1998). These are named aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2. *Aspergillus flavus* produces two toxins, AFB1 and AFB2, and *Aspergillus parasiticus* produces all four toxins (AFB_1, AFB_2, AFG_1, and AFG_2). The four compounds are distinguished on the basis of their fluorescence color under long-wave ultraviolet illumination, where B stands for blue and G for green. The subscripts relate to their chromatographic mobility. AFB_1 is usually found in the highest concentrations, followed by AFG_1, AFB_2, and AFG_2 (McLean and Dutton, 1995). Aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are hydroxylated forms of AFB_1 and AFB_2 (Figure 2). Aflatoxin B_{2a} (AFB_{2a}) and aflatoxin G_{2a} (AFG_{2a}) are 8,9-hydrated products of AFB_1 and AFG_1, respectively. These compounds are not as toxic as AFB_1 and AFG_1.

Aflatoxins are highly soluble in moderate polar solvents (e.g., chloroform and methanol), and also have some water solubility (McLean and Dutton, 1995). AFB_1 is both lipid and water soluble and these characteristics assist its accumulation and passage

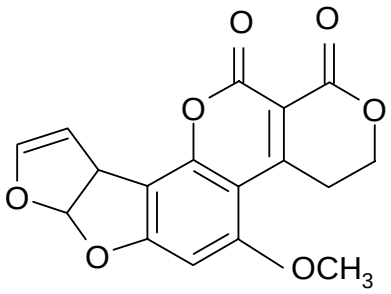
through cell membranes and into cellular organelles (Palanee et al., 2000). Aflatoxins are very heat stable and can undergo partial or no destruction under ordinary cooking



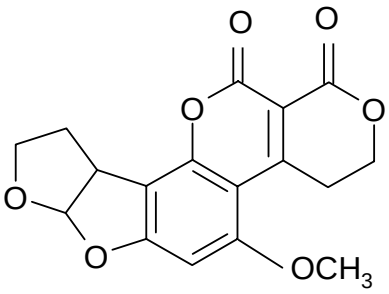
AFB_1



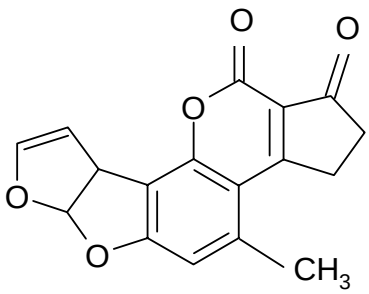
AFB_2



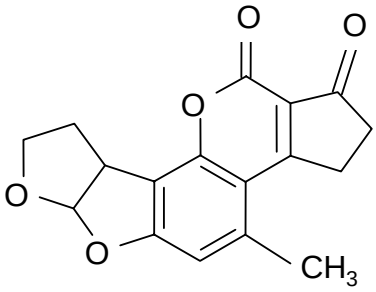
AFG_1



AFG_2



AFM_1



AFM_2

Figure 2. Structures of Aflatoxins B_1, B_2, G_1, G_2, M_1, and M_2.

conditions or during pasteurization. AFB_1 decomposes without melting at 268-269 ^oC (Beuchat, 1978). From a toxicological point of view, aflatoxin may be considered a potential threat because it can act as a potent toxin, a mutagen, a teratogen, and a carcinogen. According to epidemiological studies, there is evidence relating AFB_1 to primary liver cancer (PLC) (Li et al., 2001). Aflatoxin itself is not carcinogenic, but when ingested it can be metabolized by the body to produce an ultimate carcinogenic metabolite known as AFB_1-8,9-epoxide. The biotransformation to the epoxide is accomplished by a bioactivation system and subsequent covalent binding to DNA or proteins (Palanee et al., 2000).

2. Occurrence of Aflatoxins in Agricultural Commodities

Aflatoxins are naturally occurring toxic chemical by-products produced by the fungi *Aspergillus flavus* and *A. parasiticus*. These toxins belong to the well-known category of mycotoxins that are toxic substances produced by *Aspergillus* spp. These molds represent a threat to the safe use of numerous agricultural commodities, such as corn, cottonseed, and peanuts (Zuber et al., 1987). These secondary metabolites can result in illness or death of animals, including humans. The diseases caused by these mycotoxins are called mycotoxicoses in general and aflatoxicosis from ingestion of aflatoxin-contaminated foods and feeds. Aflatoxin-producing fungi require appropriate conditions to produce aflatoxin as a secondary metabolite. Therefore, its production is only favored by certain environmental conditions, such as temperature (>90 ^oF), humidity (>80%), the oxygen level and/or chemical characteristics of the agricultural products that serve as the substrate for aflatoxin production (Uraguchi and Yamazaki, 1978). Contamination of the above commodities commonly occurs before, during, and after

harvest. Improper storage conditions allow spores to develop and subsequently produce aflatoxins. Infections are not uniform throughout a load of feed, which makes effective sampling of feed very difficult.

One of the main reasons aflatoxins are widely distributed is that *A. flavus* is naturally found in air and soil worldwide. *Aspergillus flavus* deteriorates a number of stored crops, such as corn, cottonseed (*Gossypium herbaceum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), peanuts (*Arachis hypogaea*) and many more (Uraguchi and Yamazaki, 1978). During storage, this toxic mold grows at relatively low moisture levels. Aflatoxins are more common in grains from southern regions and are rare in northern areas of the USA (Abbas et al., 2002). However, severe drought conditions during grain fill can favor aflatoxin contamination of corn crops, creating concerns for marketing and utilizing corn. Furthermore, contamination may also occur when agricultural commodities are not promptly dried or properly stored (Betran et al., 2002).

Although aflatoxin B_1 is a ubiquitous contaminant of several classes of commodities, contamination of corn likely poses the greatest health risk to humans worldwide. This is due primarily to the importance of this commodity as a food and feed source throughout the world. Regions with higher incidence of primary hepatocellular carcinoma (PHC), where aflatoxin-contaminated corn is still a major food source for human consumption, leads to higher incidence of fungal infections in humans (Li et al., 2002). Direct economic losses resulting from the presence of aflatoxin in agricultural crops lead in reduced crop quality, yield, animal performance and reproduction capabilities, and increased incidence of diseases (Smith, 1997). Corn producers in Louisiana, Texas, and Mississippi suffered $85 to $100 million losses due to aflatoxin

contamination during 1998 (Betran et al., 2002). It has been estimated that the combined annual losses related to aflatoxins ranged from $0.5 million to over $1.5 billion in the USA alone (Robens and Cardwell, 2003). The annual losses arising from the impact of mycotoxins in the feed and livestock industries are of the order of $ 5 billion (Coker, 1998).

3. Aflatoxin Contamination of Corn

A variety of conditions favor A. *flavus* invasion of grains and subsequent production of aflatoxin. Some of these environmental conditions include daytime high temperatures of 90°F or greater, relative humidity of 80% or above, injury caused by insects, birds or hail, as well as drought stress, which predispose the crop to colonization by the fungus and aflatoxin contamination. Furthermore, rainfall at the end of the growing season postpones harvest and prevents dry-down, and lastly storage conditions with corn moisture above 13% and moderate temperatures increase the risk of aflatoxin contamination (Li et al., 2001).

Aflatoxins are chemicals produced by *Aspergillus* spp. that elicit a wide range of toxic responses in animals and humans. They are inevitably found in foods and feeds; therefore the major mode of contamination in man and animals is through ingestion. Approximately 25% of the world's food supply is contaminated by mycotoxins annually. Aflatoxins have been implicated in human diseases and for this reason the U.S. FDA regulates AFB_1 in foods. The current action level, the concentration above which the commodity is condemned for human consumption, is 20 ppb of total aflatoxins (FDA, 2000). Aflatoxin B_1 is a potent chronic or sub-chronic toxin that primarily targets the liver. The primary lesions include hemorrhagic necrosis, fatty infiltration, and bile duct

proliferation. Aflatoxin B_1 is also carcinogenic in a wide variety of animals (Li et al., 2001). As in the case following acute exposures, the major target organ is the liver, although tumors in other organs result from long-term dietary exposure to AFB_1. According to the International Agency for Research on Cancer, AFB_1 is implicated in the cause of PHC and its incidence varies throughout the world. PHC is one of the most common cancers in China, Sub Saharan Africa, the Philippines, and Thailand, and causes at least 250,000 deaths annually worldwide (Li et al., 2002).

III. Decontamination Processes of Aflatoxin-Contaminated Commodities

1. Introduction

Aflatoxin contamination can result in corn crops either before, during, or after harvesting. Proper harvest and storage practices can reduce the risk of aflatoxin contamination. Human exposure to aflatoxins may result either through direct consumption of contaminated processed or unprocessed foods or indirectly by consuming contaminated products from animals that have been fed with contaminated feeds. Therefore, prevention seems to be the most suitable approach to control human and animal exposure to aflatoxin contamination (Zuber et al., 1987). However, this approach is not always possible. Aflatoxin contamination is a worldwide unavoidable problem and as of today there are several strategies available for the detoxification or decontamination of commodities containing mycotoxins. These can be classified as chemical, microbiological, or physical. Many studies have evaluated the use of chemicals for the detoxification and decontamination of contaminated raw materials by destroying or modifying mycotoxins so as to reduce or eliminate the toxic effect. Often chemical treatments have been used in combination with physical treatments to increase the

efficacy of decontamination. A variety of chemicals (many acids, bases, aldehydes, bisulfite, oxidizing agents and various gases) can react to destroy or degrade aflatoxins effectively but most are impractical or potentially unsafe to use because of the formation of toxic residues or the effect on nutrient content, flavor, odor, color, texture, and/or functional properties of the product.

2. Ammoniation

Ammoniation is the most commonly used technique for detoxification of aflatoxins and has received considerable attention. The ammoniation process using either ammonium hydroxide or gaseous ammonia has been shown to reduce aflatoxins (100-4000 mg/kg) by up to 99% in corn, peanut meal-cakes, whole cottonseed, and cottonseed products. If the reaction is allowed to proceed to completion, the process is irreversible (Park, 1993). A high pressure/high temperature ammoniation process (80-120 ^{o}C/ 35-50 psi) for 20-60 minutes is used to remove aflatoxin from cottonseed and from cottonseed meal. The efficacy of ammoniation treatment to significantly reduce the toxicity (hepatic neoplasia, immunotoxicity) of aflatoxins has been demonstrated by feeding animals with ammonia-treated and untreated aflatoxin-contaminated corn, peanut meal and mixed feed. The states of Arizona, Texas and California permit the ammoniation of cottonseed products and Texas, North Carolina, Georgia and Alabama have approved the ammoniation procedure for aflatoxin-contaminated corn (Coker, 1998). Mexico has approved ammoniation for corn and France, South Africa, Senegal and Brazil use this procedure to lower aflatoxin contamination levels.

3. Ozonation

Ozone is a powerful oxidant which can react with several chemical compounds. Contaminated corn is treated with ozone gas for a given period of time to reduce the mutagenic potential of aflatoxin-contaminated corn. Prudente and King (2002) recently performed an evaluation study in determining the efficacy of ozone treatment to reduce aflatoxin in aflatoxin-contaminated corn. However, ozonation efficacy must be further evaluated and more research is currently performed to introduce this highly potential detoxification process as one the decontamination processes used.

CHAPTER 3
MATERIALS AND METHODS

I. Materials

Lutein standard, linoleic acid, and lipoxidase were purchased from Sigma (St. Louis, MO). The HPLC column (YMC_{30}) was a product of Waters (Mildford, MA). Whole corn samples with varying concentrations of aflatoxins were kindly provided by Louisiana Agricultural Experiment Station, Louisiana State University AgCenter and Dr. Manjit S. Kang, Department of Agronomy, Louisiana State University AgCenter (Baton Rouge, LA). All reagents were either HPLC grade or reagent grade. Corn oil was obtained from a local store and used without any further purification. Multifunctional cleanup system (MFC) (Mycosep Romer column #224) was obtained from Romer Laboratories, Inc (Washington, MO).

II. Methods

1. Corn Sample Preparations

Whole aflatoxin-contaminated corn provided by LSU agricultural center experiment station were used for preliminary analysis, whereas 20 varieties of aflatoxin-contaminated corn samples (200 g each) (Appendix A), which were provided by LSU AgCenter Department of Agronomy were used for evaluation of the designed protocol. Aflatoxin-contaminated corn (200g) was ground using a Brinkman mill (Brinkman Instruments, Westbury, NY., USA) to pass a No. 20 mesh screen. Samples were then transferred into clean plastic bags, labeled, and stored at room temperature for 24 hours until analysis.

2. Lutein Extraction

Aflatoxin-contaminated corn samples (50g) were treated with acetone using 1:3 corn sample to solvent ratio. The mixture was allowed to shake for one hour in the absence of light to prevent lutein decomposition. The acetone-treated samples were filtered using Whatman No. 4 filter paper. The filtrate was saved and the extraction process was repeated. The second filtrate was combined with the filtrate from the first extraction. The filtrate was then evaporated using a Buchi Rotavapor R-200 evaporator (Brinkman Instruments Inc., Westbury, NY) and saponification was achieved by dissolving the extract in 10% potassium hydroxide in methanol. The samples were allowed to shake overnight in the absence of light followed by extraction of lutein from the mixture using hexane: ethyl ether (1:1) in a separatory funnel. Extraction was repeated until the orange color of the solution had faded or disappeared completely. The lower aqueous phase was washed with hexane/ethyl ether solution for re-extraction until the aqueous phase was colorless. All hexane: ethyl ether extracts were combined and evaporated. Extracts were dissolved in 20 mL of methanol: methyl-*tert*-butyl-ether (95:5 dilution) and the solution was passed through a 0.4μm PTFE filter membrane (Millipore, Bedford, MA, USA) for HPLC analysis or stored at -20 ^{o}C until use (Figure 3).

3. Lutein Determination from Corn Extracts

For standard curve determination lutein standards were prepared in parts per million (ppm). Lutein standard (dried powder) was dissolved to the desired concentration using the mobile phase solvent, MTBE: MeOH (5:95). The known concentration of lutein standard mixture was used to prepare standard solutions of desired concentration. Three milliliters of corn pigment extract and the standards were filtered through a 0.2 μm PTFE

filter membrane. The filtered samples were injected into an YMC_{30} carotenoid 3µ, 4.6 x

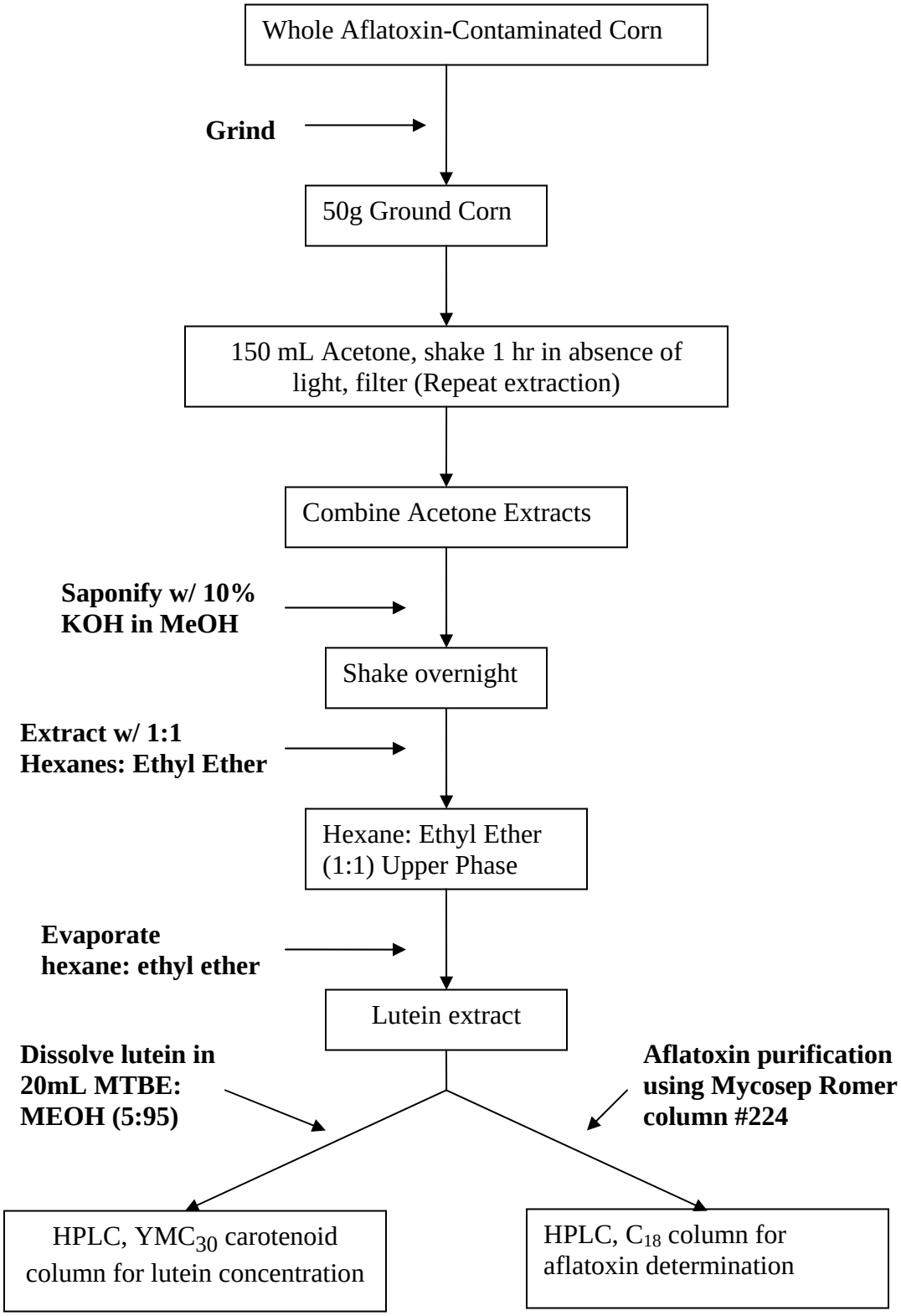


Figure 3. Flow diagram of the extraction process.

250 mm HPLC column. The HPLC separation was carried out using a Waters Model 600E solvent delivery system fitted with a model 717A plus autosampler, a Model 486 tunable absorbance detector and Millennium 32 chromatography manager processor (Milford, MA). The flow rate was 1ml/min, detection was at 450nm, the injection volume was 20ul, and separation was isocratic using MTBE: methanol (5:95) as the mobile phase, with a total separation time of 30 minutes.

Peaks on a chromatograph were identified by comparing their retention times and spectra with those of lutein standards. A calibration curve was constructed by plotting the area under the peak vs. lutein standard concentration between 0 and 100 ppm. Lutein concentration in corn samples was determined by using a regression equation obtained from the calibration curve.

4. Enzymatic Treatment of Extracted Lutein Residue

Extracted samples with approximately 0.55mg of lutein were dissolved in approximately 10 mL of corn oil for further treatment with lipoxidase (LOX). The incubation mixture contained 1.0 mL Tris-HCl (pH 7.2), 50 μg of lipoxidase, and 50 μM AFB_1 in 20 μl DMSO. Sample extract in corn oil and DMSO solution were combined in a 1:1 ratio. After a 3-minute pre-incubation at 37 ^{o}C, the reaction was initiated by the addition of the desired polyunsaturated fatty acid and incubated for 2 hours at 37^{o}C. When the enzyme treatment was complete, the samples were divided in two aliquots. One aliquot was used for aflatoxin determination using a Mycosep Romer column #224 as described below (Figure 4). The other aliquot was saponified, lutein was extracted and analyzed by HPLC as described above. Samples were stored at -20 ^{o}C for HPLC analysis for aflatoxin determination as described below.

5. Aflatoxin Purification

The samples were analyzed following the approved Multifunctional Column (Mycosep) method of the Association of Official Analytical Chemists (AOAC) (AOAC, 2000). The treatment protocol included a set of control corn samples, clean corn and aflatoxin-contaminated corn. Fifty grams of clean ground corn was mixed with 100 ml of acetonitrile: water (9:1) solution. The samples were placed on a shaker in the absence of light for 30 min and then filtered using Whatman No.4 filter paper. The same procedure was performed with the untreated aflatoxin-contaminated corn. Control samples and treated samples were purified through the Multifunctional cleanup column (Romer Labs, Inc.). Approximately 2 ml of the samples were placed in the culture tube and the flanged-end of the column was pushed into the extract, letting extract pass through the column. An aliquot of the purified extract is used for quantification of aflatoxins as described below.

6. Quantification of Aflatoxins by High Performance Liquid Chromatography

The samples were analyzed according to the AOAC-approved Multifunctional Column (Mycosep) Column (AOAC Official Method 994.08, 2000). Initially, aflatoxins were derivatized by adding a 200 μl aliquot of the purified extracts into an HPLC auto-injector vial (Waters, Milford, MA), and 700 μl of trifluoroacetic acid derivatizing reagent [distilled water: trifluoroacetic acid: glacial acetic acid (7:2:1)]. The samples were placed in a water bath at 65 ^{o}C for 8.5 min. The vials were cooled in an ice-water bath. The samples were placed in the HPLC autosampler (Waters 717). The injection volume was 50ul and the total separation time was 15 min. Aflatoxin levels were determined by using a Waters 510 HPLC (Waters Corp., Milford, MA) equipped with

Waters 470 fluorescence detector (360 nm excitation and 440 nm emission), and a NovaPak C_{18} reverse phase column (Waters, 3.9 mm x 150 mm) using water: acetonitrile (8:2 v/v) as a mobile phase with a flow rate of 2 ml/min. Approximate retention times for aflatoxin G_1, B_1, G_2, and B_2 were 2.2, 3, 5.5, and 8.3 min, respectively. Aflatoxin concentrations were calculated by using a plotted standard curve that automatically reports aflatoxin concentrations by the Millennium Chromatograph Manager Software (Waters Inc., Milford, MA).

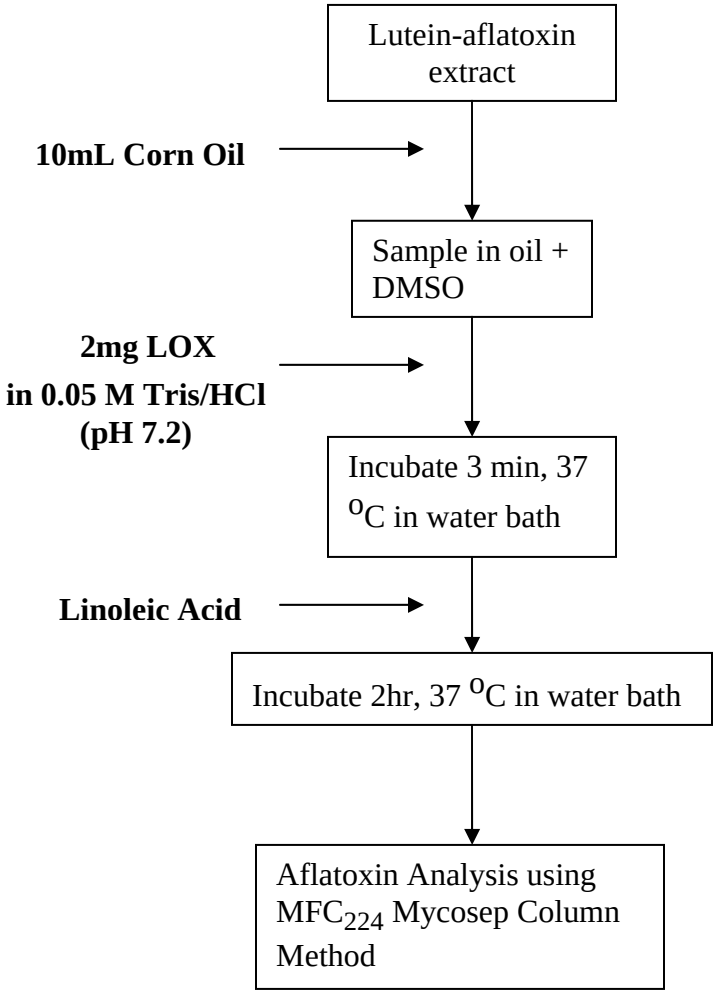


Figure 4. Flow diagram of enzymatic treatment of aflatoxin-contaminated lutein.

7. Evaluation of Lutein Stability by High Performance Liquid Chromatography.

The samples following the enzyme treatment were extracted using hexane: ethyl ether (1:1) following the extraction procedure as described above to recover the lutein present. Following extraction, the solvent was evaporated using a Buchi Rotavapor R-200 evaporator (Brinkman Instruments Inc., Westbury, NY). The isolated lutein extract was dissolved in MTBE: MeOH (5:95) for HPLC analysis as described in materials and methods to determine the presence of lutein.

CHAPTER 4
RESULTS AND DISCUSSION

I. Identification of Lutein in Aflatoxin-Contaminated Corn

The elution time of the lutein standard using the YMC_{30} carotenoid column and reverse-phase chromatography was less than 30 min; the elution profile is shown in Figure 5. To obtain satisfactory lutein separation in the column, lutein should be completely extracted and released from its ester form. This was accomplished by saponification that also eliminated contaminating substances such as lipids and proteins that could potentially plug the carotenoid column (Moros et al., 2002).

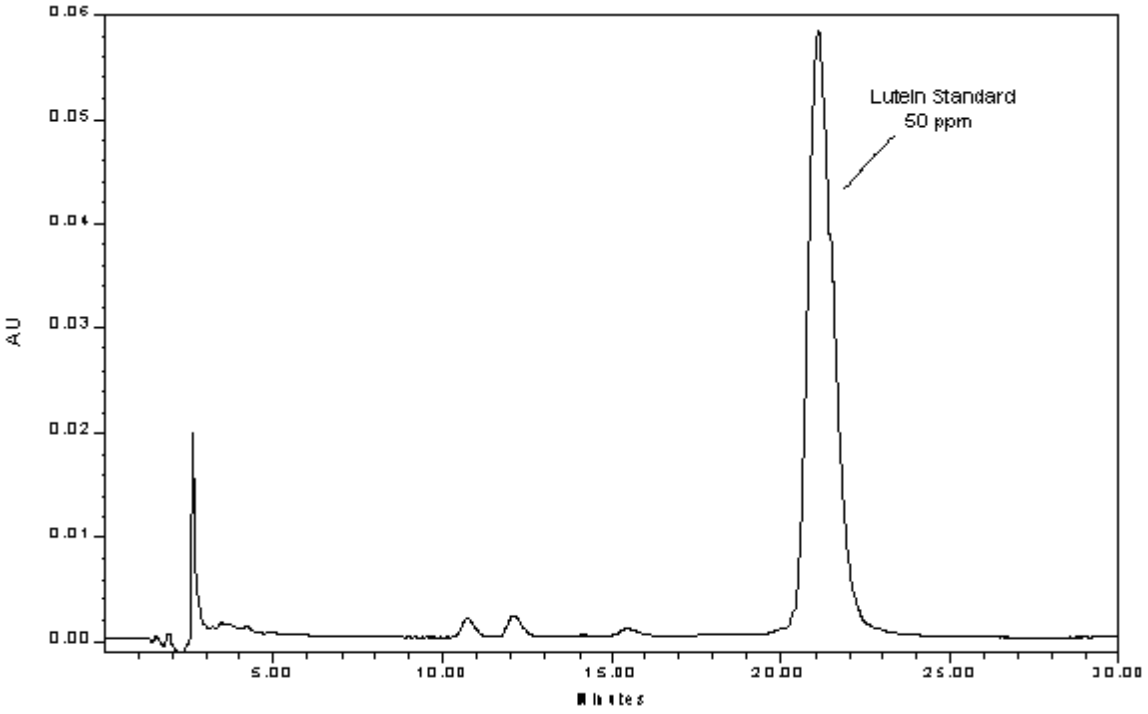


Figure 5. Elution profile of lutein standard at a concentration of 50 ppm.

Lutein peak was well separated by the C_{30} column. Lutein standards of varying concentrations in parts per million varying from 25 to 100 ppm were eluted and separated for standard curve determination and the content of lutein in the samples was calculated

by comparing the peak area with that of standard lutein. Aflatoxin-contaminated corn samples were analyzed for lutein by HPLC; the chromatogram in Figure 6 illustrates the elution of lutein with a retention time of approximately 21 min. As described previously, lutein from aflatoxin-contaminated corn was isolated by acetone extraction, saponification, and hexane: ethyl ether extraction followed by drying, solubilization in MTBE: MeOH, and HPLC separation. From 1.61mg of lutein measured in 100g of aflatoxin-free corn, 1.10 mg of lutein was measured in 100g from aflatoxin-contaminated corn samples as shown by HPLC analysis (Table 3). The amount of lutein recovered appears to be decreased and this can be explained based on instability of the carotenoid isomers which can be accelerated during aging and heating (Dachtler et al., 1998).

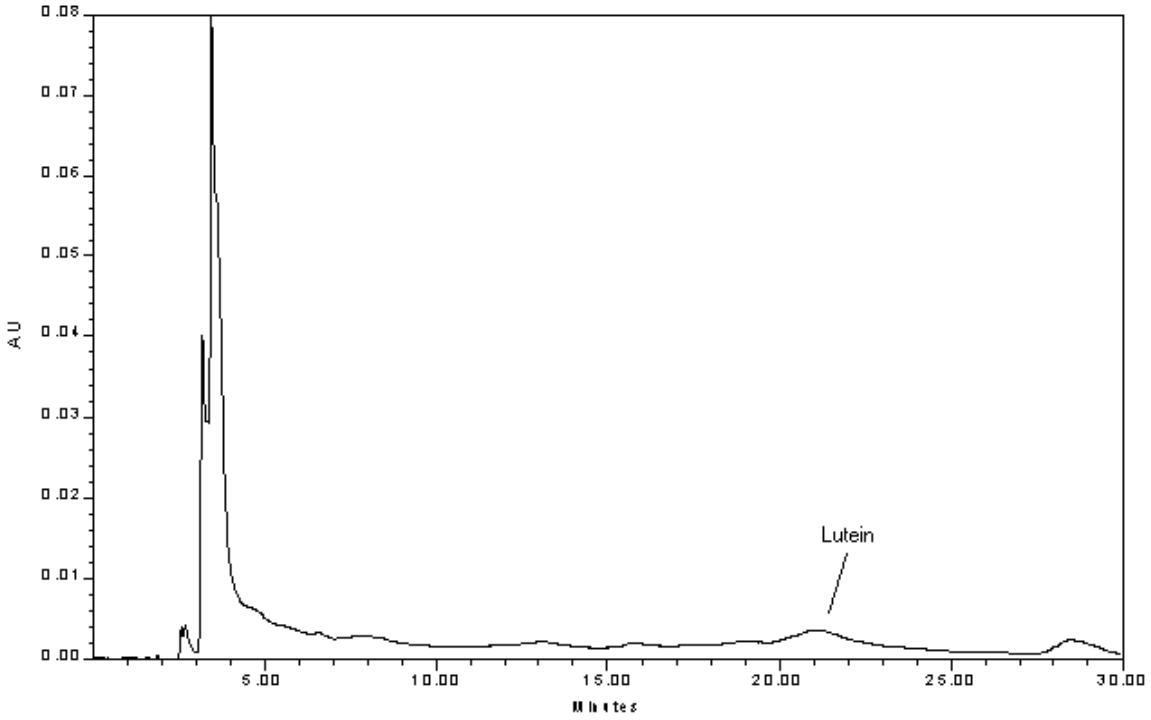


Figure 6. Lutein isolated from aflatoxin-contaminated corn.

Repetitive analysis of 20 varieties of aflatoxin-contaminated corn samples using HPLC demonstrated uniform lutein content (Appendix A). The mean concentration of lutein from the samples

Table 3. Lutein Concentrations from HPLC Analysis.

Sample	Lutein concentration (mg/100g corn)*
Aflatoxin-free corn	1.61 ± 0.06
Aflatoxin-contaminated lutein extract	1.10 ± 0.07
Enzyme-treated lutein extract	0.97 ± 0.04

* Values are mean concentrations ± SD.

analyzed by HPLC before the lipoxidase treatment was 1.10mg/100g (dry wt.) of aflatoxin-contaminated corn and 0.07 standard deviation (Table 3).

II. Enzymatic Treatment of Aflatoxin-Contaminated Corn and Determination of Aflatoxins

Aflatoxin levels were determined by analyzing the samples following the Multifunctional Column (Mycosep) method (Prudente and King, 2002). The samples were enzymatically treated and analyzed with HPLC following the cleanup procedure as described under materials and methods. Figure 7 represents the elution profile of a mixture of aflatoxin standards with a concentration of 100 ppb. Four peaks were identified with AFG_1 eluting first, followed by AFB_1, AFG_2, and lastly AFB_2. Approximate retention time for AFG_1, AFB_1, AFG_2, and AFB_2 were 2.4, 3.3, 6, and 9.2 min, respectively (Figure 7). Aflatoxin-free corn was also analyzed under the same conditions and no aflatoxin peaks were identified on the chromatogram as shown in Figure 8. This indicated that no aflatoxin was present or it was present at non detectable levels. A representative aflatoxin-contaminated corn sample was extracted with

acetonitrile: water (9:1) and analyzed for aflatoxin via HPLC. The HPLC profile of aflatoxin separated by chromatography is given in Figure 9. The two peaks identified were associated with AFB_1 and AFB_2, respectively. No peaks were identified for AFG_1 and AFG_2. The retention times of AFB_1 and AFB_2 were 3.4 and 9.3 min, respectively. A similar representative of aflatoxin-contaminated corn was extracted for lutein using acetone, saponification, hexane: ethyl ether extraction, evaporation, dissolution in acetonitrile: water (9:1), mycosep column purification of aflatoxin followed by HPLC analysis of aflatoxins. Chromatographic analysis of aflatoxin-contaminated lutein extract also showed peaks of AFB_1 and AFB_2 at retention times of 3.4 and 9.1 min, respectively (Figure 10).

Aflatoxin concentrations were calculated using the Millenium Chromatography Manager Software (Waters Inc., Miliford, MA) (Table 4). The results of aflatoxin concentrations demonstrate that the aflatoxin forms present in higher amounts were AFB_1 and AFB_2 (Table 4). McLean and Dutton (1995) reported that AFB_1 is usually found in the highest concentrations in commodities. Based on the results of the aflatoxin concentrations, the enzyme treatment eliminated the aflatoxin present in corn to non-detectable levels following enzyme treatment (Figure 11). In a preliminary study, the enzyme treatment was also performed with triplicate samples containing 2.5 times higher concentration of aflatoxin than the samples reported in Figure 11 in order to evaluate the efficiency of the enzyme treatment. The HPLC profile from these samples is shown in Figure 12 and shows complete absence of peaks associated with AFB_1 and AFB_2. Lipoxidase (LOX) is an inducible enzyme and has the ability to epoxidize AFB_1 in the presence of linoleic acid and other polyunsaturated fatty acids (Roy and Kulkarni, 1997).

LOX has the ability to either completely eliminate aflatoxins or convert aflatoxins to the 8, 9-epoxide. Twenty varieties of aflatoxin-contaminated corn with varying amounts of aflatoxins and lutein were analyzed in duplicate as described above for lutein and aflatoxin determination. The results of the HPLC profiles of aflatoxins indicated no detectable levels of aflatoxins in enzyme treated samples (Figure 11) and an average lutein recovery of 0.97 ± 0.04 mg/100 g corn (dry wt.). Figure 11 is a representative chromatogram of the twenty samples analyzed in duplicate.

Table 4. Aflatoxin Concentrations from HPLC Analysis.

Sample	Aflatoxin concentrations (ppb)			
	AFG_1	AFB_1	AFG_2	AFB_2
Aflatoxins standard (100 ppb)	36.3	36.4	9.5	9.6
Aflatoxin-free corn	N.D.	N.D.	N.D.	N.D.
Aflatoxin-contaminated corn	N.D.	4888.0	N.D.	368.0
Aflatoxin-contaminated lutein extract	N.D.	1136.0	N.D.	93.0
Enzyme-treated lutein extract	N.D.	N.D.	N.D.	N.D.

N.D. = Non-detected

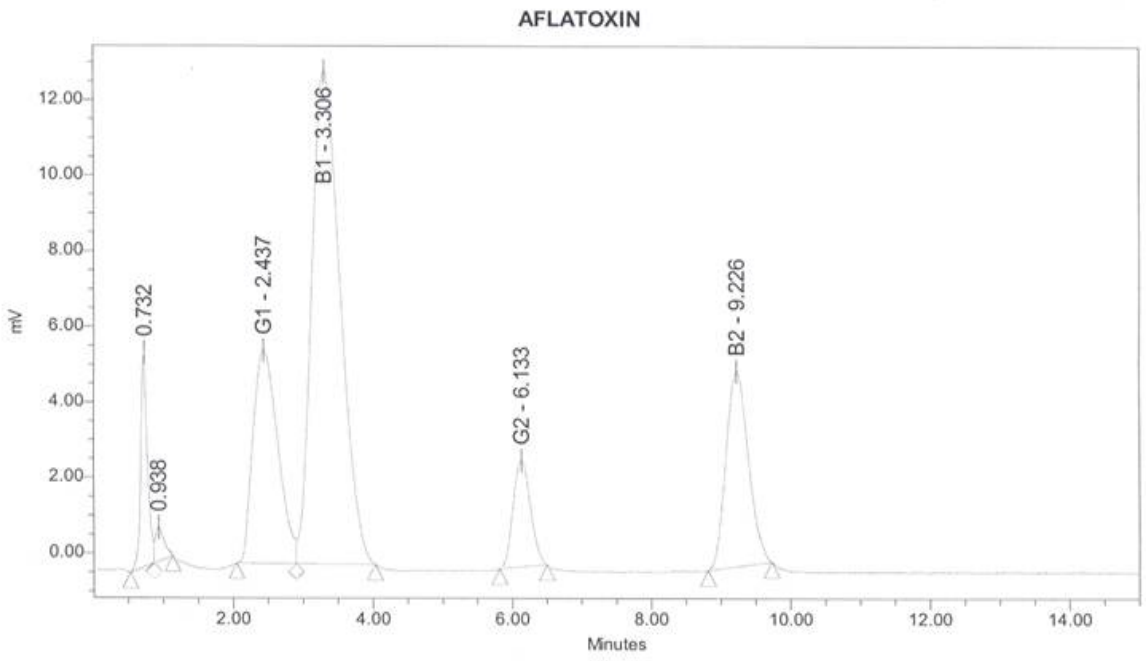


Figure 7. Aflatoxin standard at 100 ppb

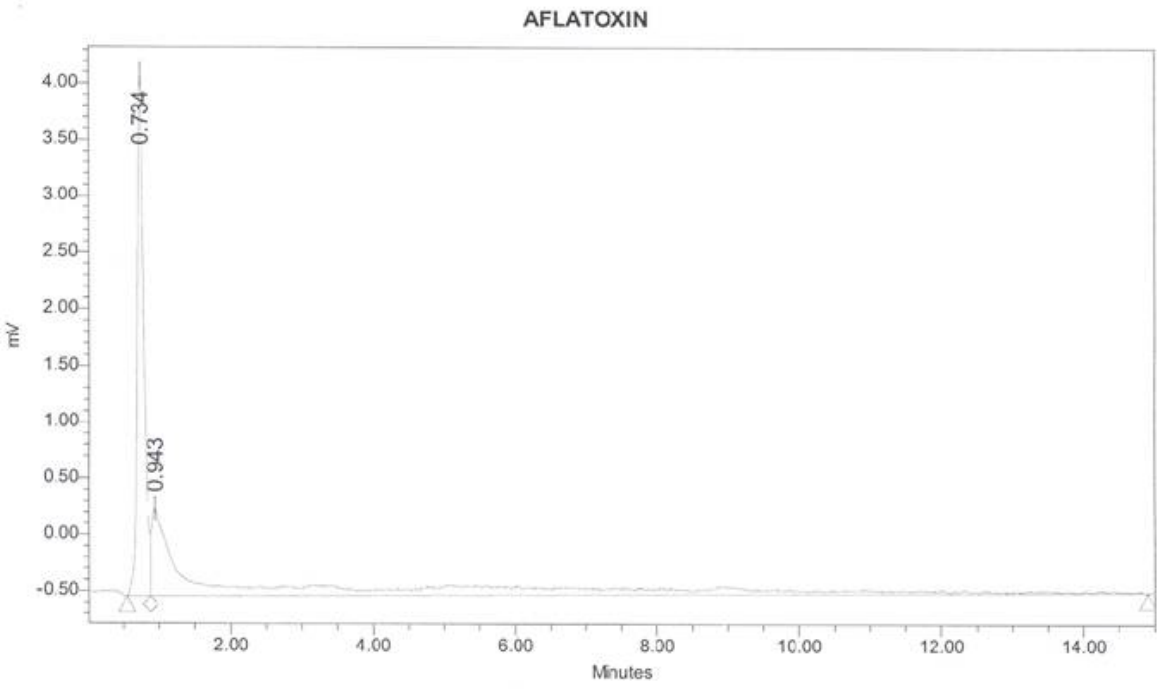


Figure 8. Aflatoxin-free corn

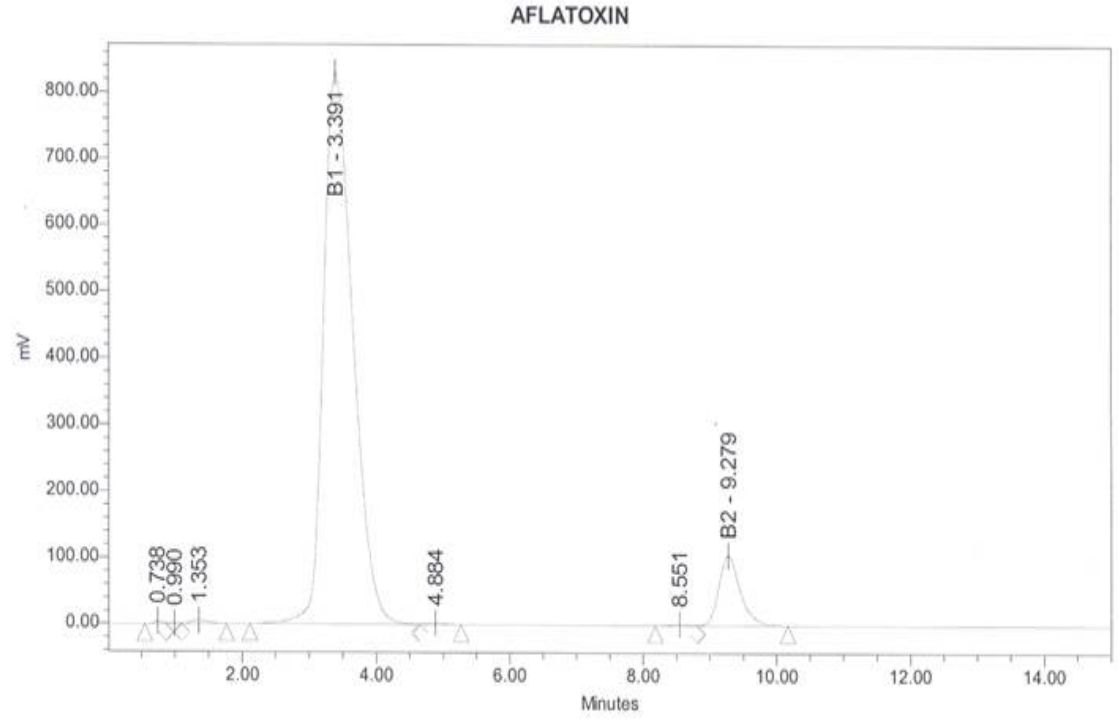


Figure 9. Aflatoxin-contaminated corn

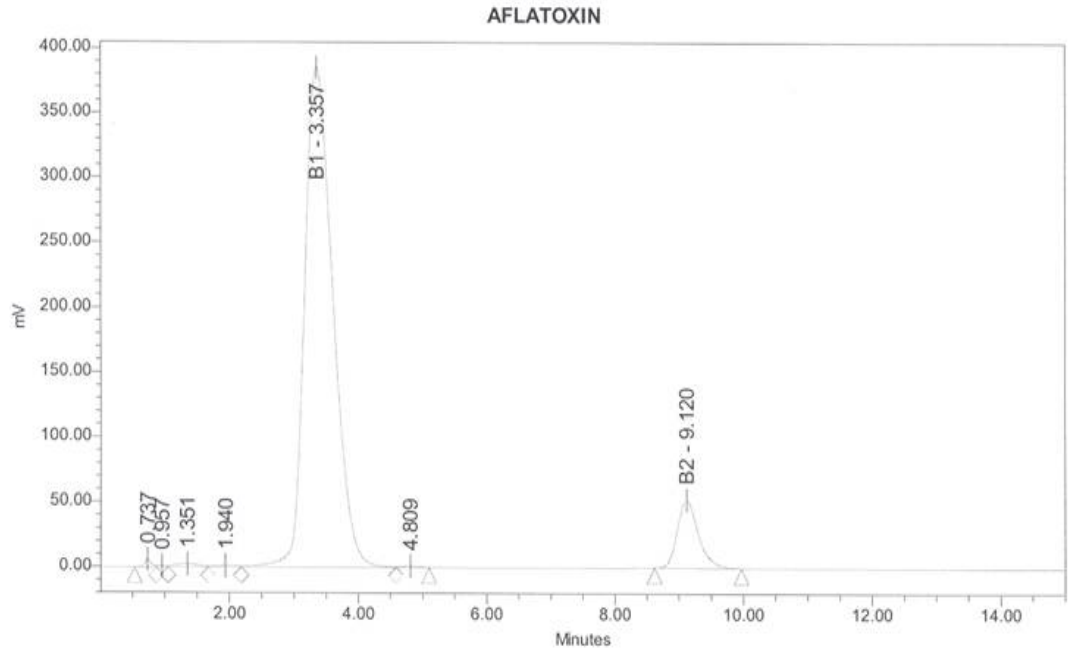


Figure 10. Aflatoxin-contaminated lutein extract

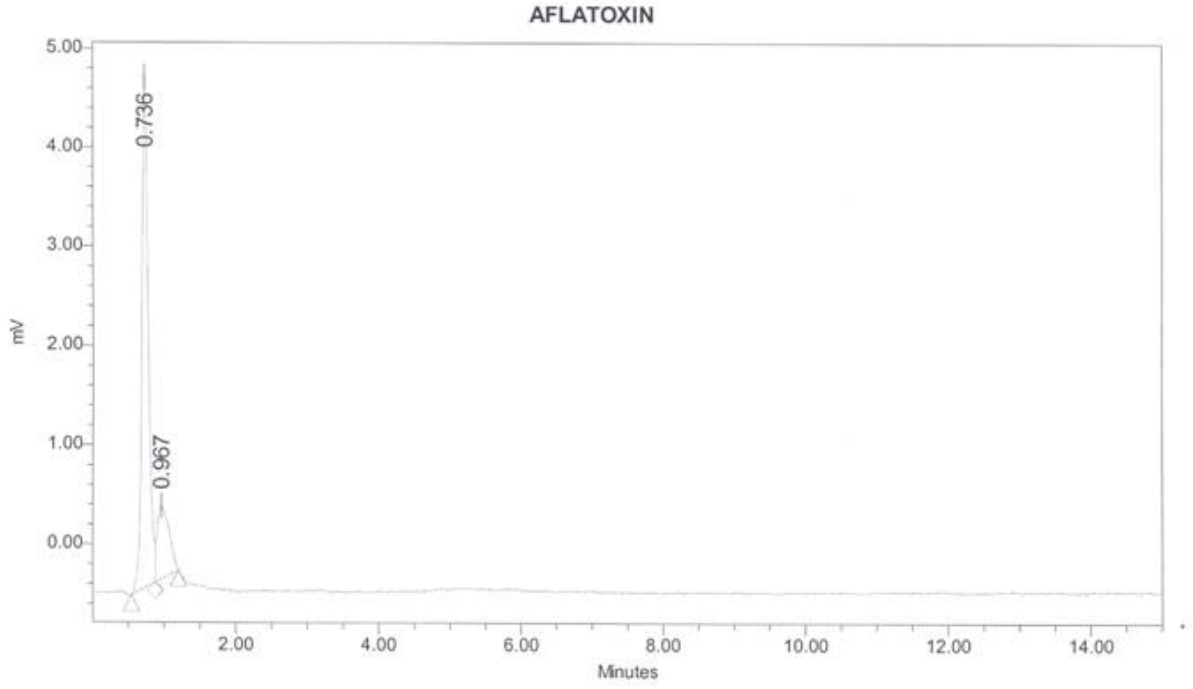


Figure 11. Enzyme-treated contaminated lutein extract showing little or no aflatoxin.

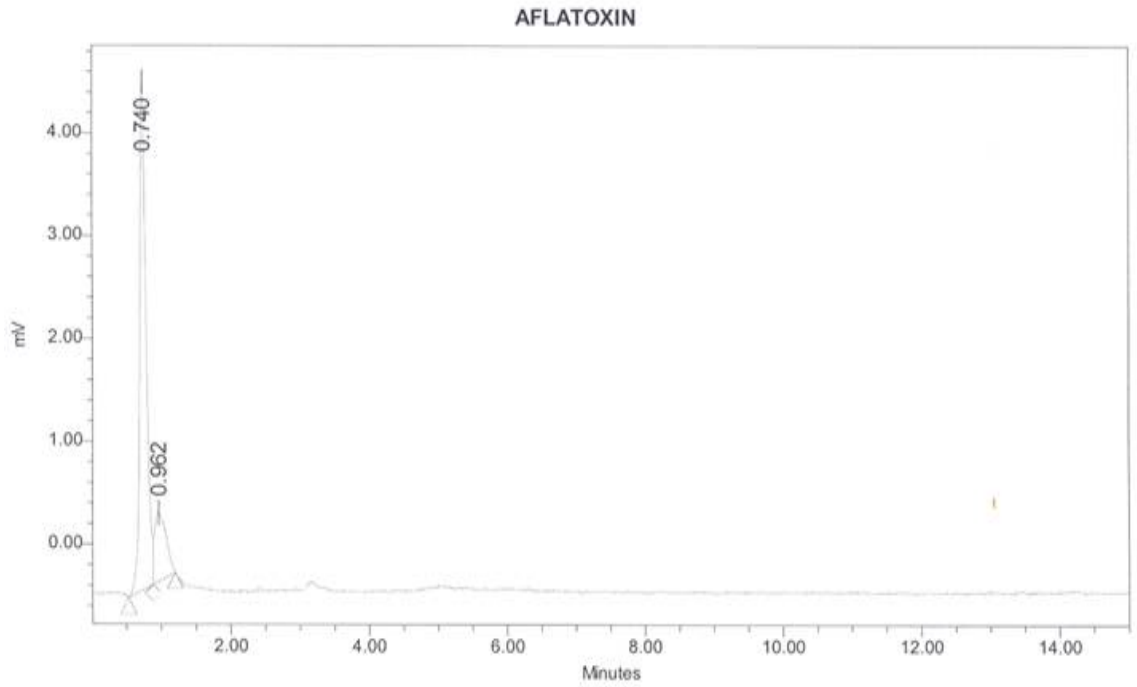


Figure 12. Enzyme-treated contaminated lutein extract (2.5 times concentration).

III. Lutein Stability upon Enzyme Treatment

The objective of this project was the isolation of aflatoxin-free lutein from aflatoxin-contaminated corn. The stability of lutein was evaluated by HPLC YMC_{30} carotenoid column after the enzyme treatment. The HPLC profile of lutein isolated from the enzyme-treated sample indicates the presence of a peak associated with lutein at ~21 min (Figure 13) eluted at approximately 21.0 min compared to the lutein standard as shown in Figure 5. The small peaks that appear at retention times approximately 15.5 and 28.5 min are probably the result of lutein degradation. They can be representatives of xanthophylls, such as all *trans* neoxanthin, 9'-cis-neoxanthin, and others, in the presence of lutein, which was identified by mass spectroscopy from extract of the green vegetables (Khachik et al., 1986).

Although no other confirmatory test, such as LC/MS, was performed to ascertain the presence of lutein, the retention time and the specificity of the column identified lutein. For further confirmation purposes, the lutein samples after the enzyme treatment were spiked with different concentrations of lutein standard. The spiking procedure was performed to confirm that the peak identified at a retention time of 21.0 min was in fact lutein. As shown on Figure 14, the lutein peak when spiked with lutein standard with a concentration of 12.5 ppm was identified at the same retention as previously. As the lutein standard concentration was increased the lutein peaks were more evident.

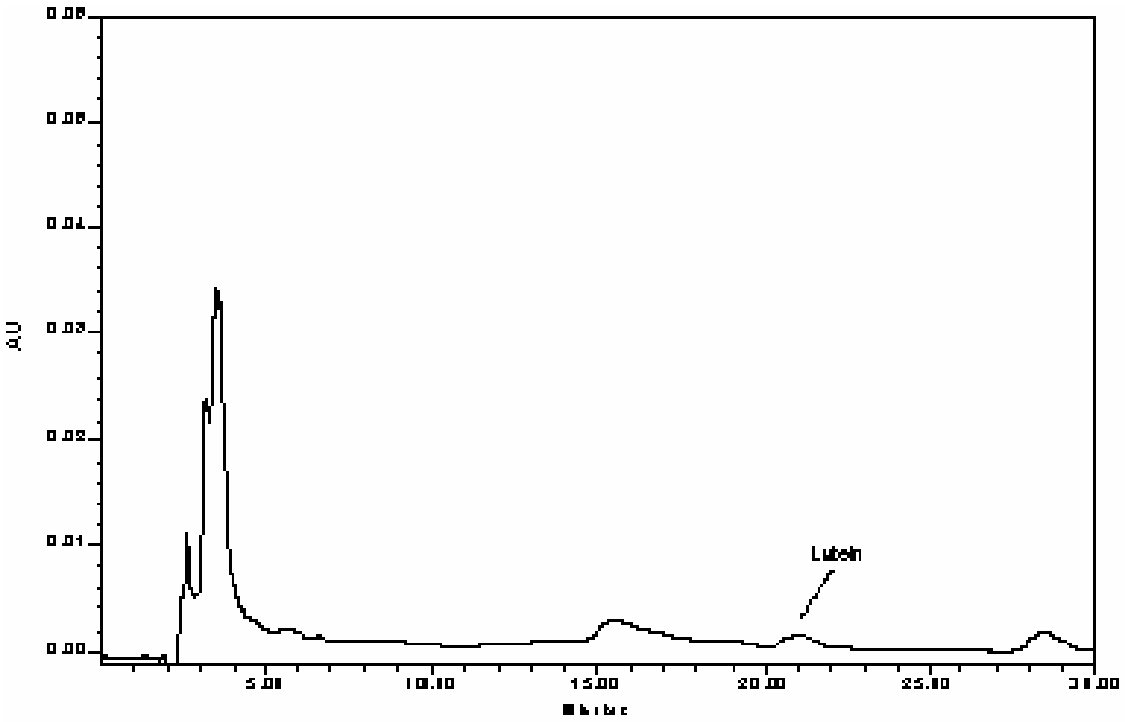


Figure 13. HPLC profile of lutein for enzyme-treated corn sample

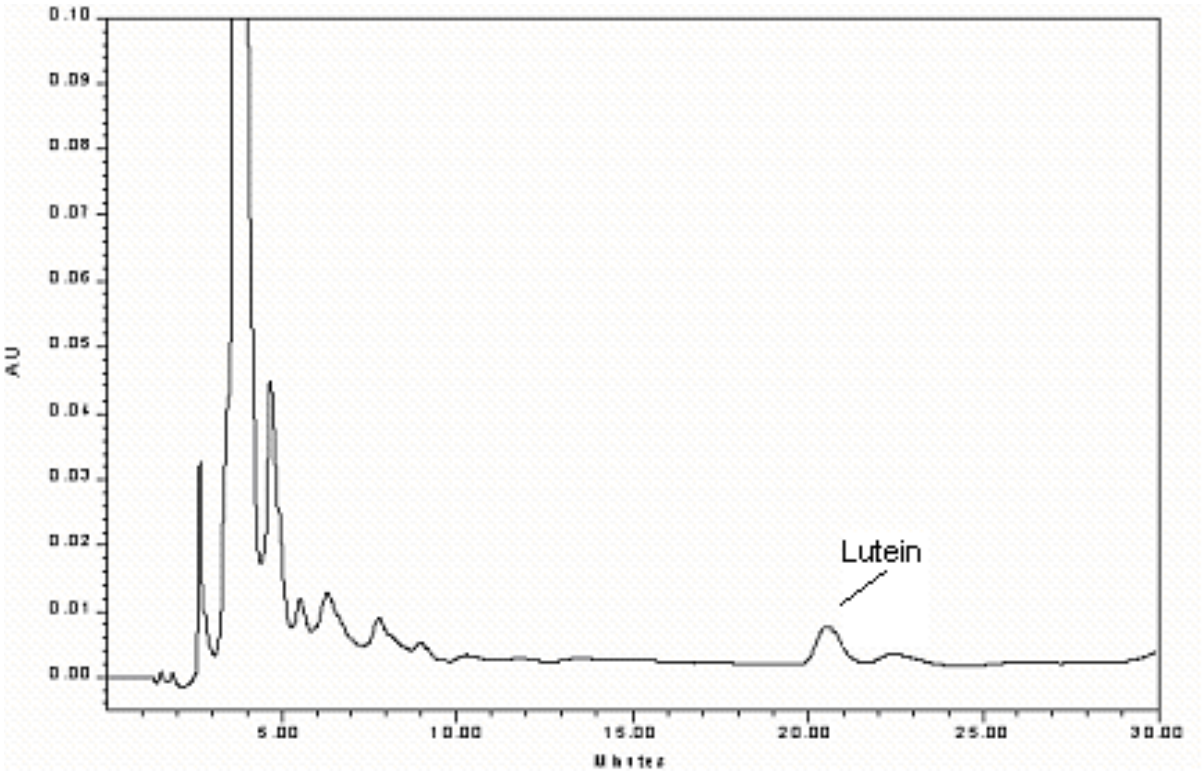


Figure 14. HPLC profile of enzyme-treated lutein spiked with 12.5 ppm of standard lutein.

Figure 15 and Figure 16 show the lutein peaks after spiking the enzyme treated samples with 25 and 50 ppm lutein concentrations, respectively. All three spiked samples were eluted at a retention time of approximately 21.0 min. From 1.10mg of lutein in 100 g of aflatoxin-contaminated corn measured before enzyme treatment, 0.97 mg of lutein in 100 g of corn was recovered following aflatoxin displacement. This method demonstrated that at least 88% lutein was stable following the enzyme treatment. Analytical techniques used in this work could not allow the identification of the 12 % lutein lost during enzyme treatment.

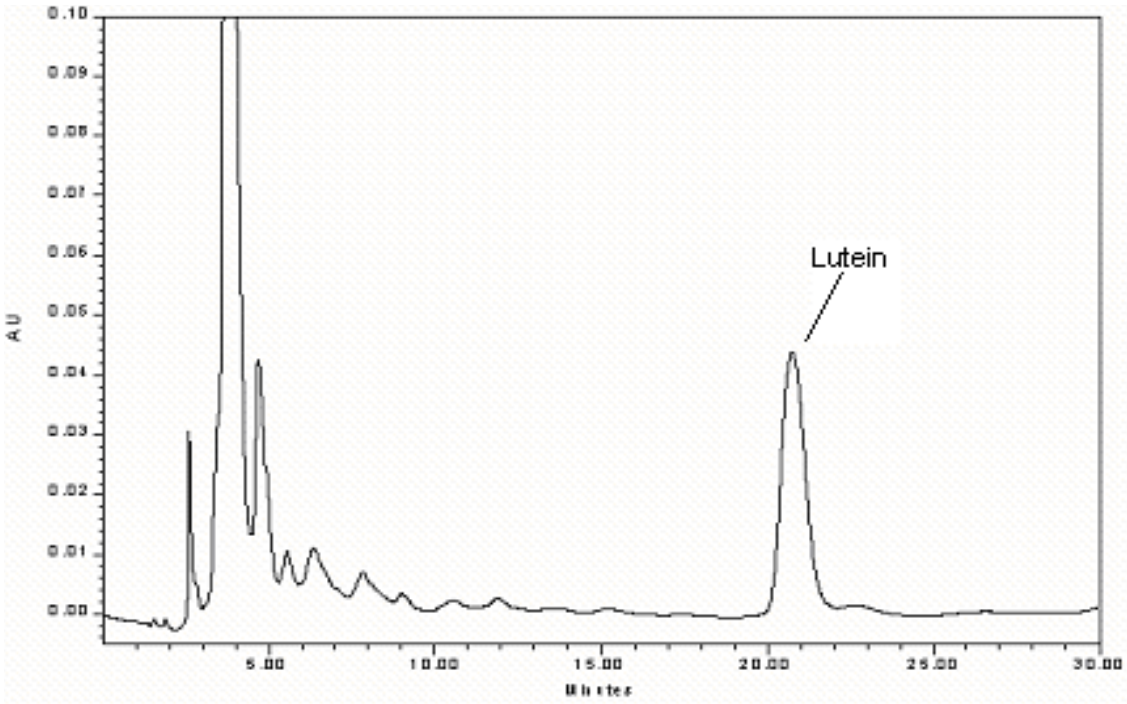


Figure 15. HPLC profile of enzyme-treated lutein spiked with 25 ppm of standard lutein.

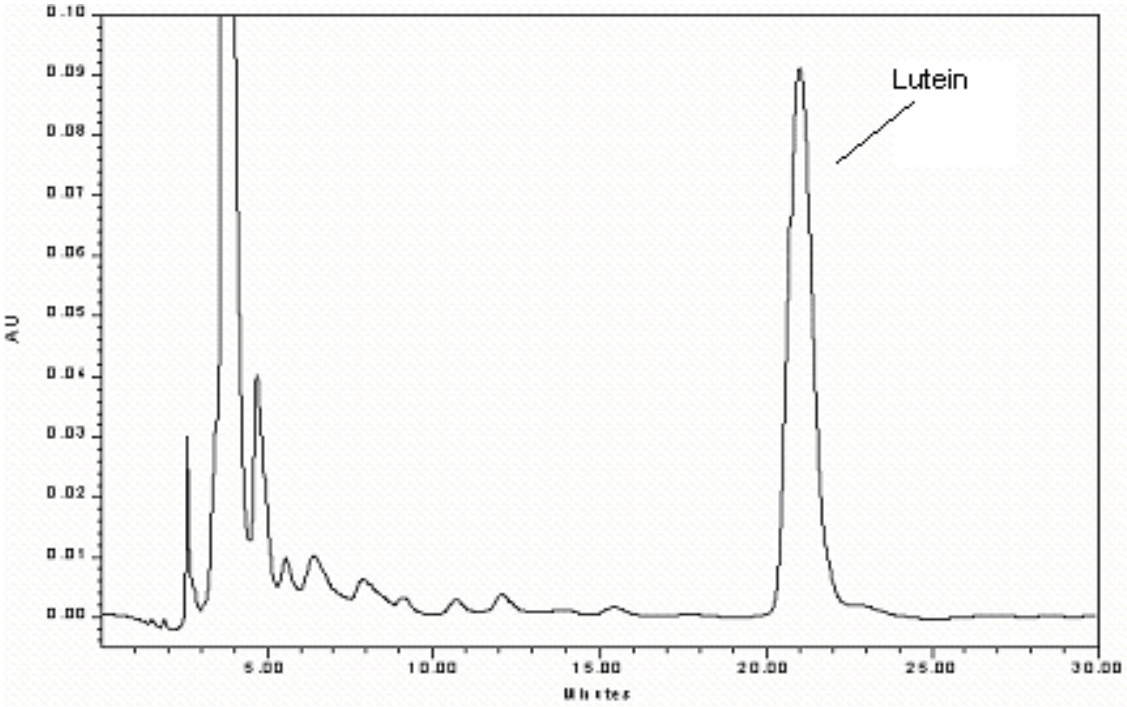


Figure 16. HPLC profile of enzyme-treated lutein spiked with 50 ppm of standard lutein.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The present study was designed to isolate aflatoxin-free lutein from aflatoxin-contaminated corn and evaluate lutein stability following the enzyme treatment process in an attempt to provide value to aflatoxin-contaminated corn other than waste.

Initially, aflatoxin-contaminated lutein was extracted from aflatoxin-contaminated corn using acetone extraction, saponification in 10% potassium hydroxide in methanol, hexane: ethyl ether extraction, and homogenized in MeOH: MTBE (95:5). Part of the aflatoxin-contaminated lutein extract was treated with lipoxidase in Tris-HCl buffer and incubated for 2 hours at 37 ^{o}C. Chromatographic analysis using HPLC YMC_{30} column was performed to determine the lutein content in aflatoxin-contaminated corn prior to and following the enzyme treatment of the samples. The results from the HPLC chromatograms showed a mean lutein concentration of 1.10 mg/100g (dry wt.) of contaminated corn prior to enzyme treatment, where following the enzyme treatment the amount of lutein recovered was approximately 0.97 mg/100g (dry wt.) of corn.

In 1998, 20% of the 50 million bushel crop (280 million tons) that had aflatoxin levels between 20 and 150 ppb was sold at a discounted price and another 4% was abandoned because it contained more than 150 ppb of aflatoxin levels (Robens and Cardwell, 2003). An estimated 67.2 million tons of aflatoxin-contaminated can allow extraction of 650 million grams of lutein. At the present level, corn lutein would be competitive because other value-added products will be produced from corn.

Chromatographic analysis based on the Multifunctional Column (Mycosep) method was used to determine the aflatoxin levels before and after the enzyme treatment. According to the results, the aflatoxin levels in contaminated corn were eliminated or

reduced to non-detectable levels after the lipoxidase treatment. The effectiveness of the treatment was evaluated by using 20 varieties of aflatoxin-contaminated corn samples and it was shown that lipoxidase treatment was reproducible and effective. The stability of lutein following the enzyme treatment was evaluated by HPLC and it was shown that 88% of lutein was stable after enzyme treatment.

A literature search that combined words such as “lutein and cottonseed and aflatoxins,” “lutein and wheat and aflatoxins,” “lutein and peanut and aflatoxins” using Medline and Agricola database produced no documents. Therefore, there is a possibility of extending the research to other oilseeds that may be susceptible to aflatoxin contamination. An investigation of the potential of this enzyme treatment to such oilseeds may assist in reducing or eliminating aflatoxin contamination in oilseeds.

The present project has shown that corn growers and processors may generate additional income from aflatoxin-contaminated corn because aflatoxin-free lutein can be isolated from this traditionally discarded agricultural commodity and may be used as a functional food or in health-enhancing products because of the positive role of lutein in human health. The overall significance of this research is that, if approved by FDA, corn growers can still sell aflatoxins-contaminated corn at a competitive price since almost all the value-added products from corn can be recovered aflatoxins-free and more lutein will be available for disease prevention.

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APPENDIX A. LUTEIN CONCENTRATION OF TWENTY AFLATOXIN - CONTAMINATED CORN SAMPLES ANALYZED BEFORE ENZYME TREATMENT

Sample	Entry #	Pedigree	Lutein mean concentration from aflatoxin-contaminated corn (mg/100g corn)
Y101	11	AR16026: N12	1.20 ± 0.03
Y106	6	UR10001: S18	1.19 ± 0.09
Y107	7	BR52051: S17	1.18 ± 0.01
Y123	16	DKB844: N11b	1.16 ± 0.02
Y112	12	ANTIG01: N16	1.15 ± 0.06
Y104	8	ANTIG03: N12	1.13 ± 0.04
Y105	4	CH05015: N12	1.13 ± 0.07
Y165	51	CHIS775: S1911b-327-1-B	1.13 ± 0.02
Y148	31	GT-mas:gk	1.12 ± 0.02
Y103	18	PRICGP3: N1211c	1.11 ± 0.005
Y262	33	UR13085: N0215-14-1-B	1.10 ± 0.04
Y111	2	AR13026: S15	1.10 ± 0.03
Y108	14	CH05015: N15	1.09 ± 0.05
Y110	13	BR51675: N0620	1.08 ± 0.045
Y102	24	UR10001: S1813	1.07 ± 0.03
Y127	15	CHIS775: N1920	1.05 ± 0.01
Y249	51	CHIS775: S1911b-327-1-B	1.05 ± 0.01
Y109	22	AR16035: S02	1.04 ± 0.005
Y264	44	FS8B(T): N1802-45-1-1SIB-B-B	1.03 ± 0.01
Y113	19	PRICGP3: N1218	1.02 ± 0.00

VITA

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